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RESEARCH

Recurrent Selection to Alter Grain Phytic Acid Concentration and Iron Bioavailability

Alyssa W. Beavers, A. Susana Goggi, Manju B. Reddy, Adrienne Moran Lauter, and M. Paul Scott*

ABSTRACT

Iron is an important micronutrient and Fe deficiency is a global health concern. Phytic acid inhibits Fe absorption and cannot be digested by monogastric livestock or humans. High phytate concentration in staple crops may be one of the contributing factors for the high incidence of anemia in developing countries because of its inhibiting effect on Fe absorption. In seeds, it serves as the main storage compound for P. Low phytic acid mutants (*lpa*) in maize (*Zea mays* L.) have improved Fe bioavailability, but they have poor germination. Our objective was to develop both low phytic acid (LPA) and high phytic acid (HPA) maize populations using recurrent selection and to compare seed quality and Fe bioavailability among the HPA and LPA populations and *lpa* mutant lines. Three cycles of selection were performed in two broad-based synthetic populations, BS11 and BS31. The resulting HPA and LPA populations were significantly different in phytic acid concentration in the BS11-derived populations ($P < 0.05$) but not in the BS31-derived populations ($P > 0.05$). The BS11LPA maize population had improved seed germination (13–16%; $P < 0.05$), and Fe bioavailability was not statistically different ($P > 0.05$) than the *lpa* mutant inbred lines. We conclude that recurrent selection for phytic acid levels may be a viable approach for improving Fe bioavailability of grain while maintaining seed quality.

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Abbreviations: G × E, genotype × environment interaction; HPA, high phytic acid; HPLC, high-performance liquid chromatography; LPA, low phytic acid; TCA, trichloroacetic acid.

PHYTIC ACID (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate) is the most abundant form of phosphate in seeds (Bewley and Black, 1994). It serves as the main storage molecule for P, providing 75% of P in seeds (Raboy, 2009). Phytic acid in seeds is found as phytate, a mixed salt of phytic acid complexed with cations, including K, Mg, Ca, Zn, and Fe (Kumar et al., 2010); among them, Fe and Zn are of nutritional concern in humans. Phytate forms phytate mineral complexes that are not readily absorbable in the human gut. Since humans lack phytase enzyme in the gut, the mineral complexes are not hydrolyzable and thereby their absorption is reduced.

Phytate is also indigestible by monogastric livestock, including swine and poultry, and as a result, it passes undigested into the feces of these animals (Cromwell and Coffey, 1991). This causes environmental problems when run off from livestock operations leads to eutrophication of waterways (Bouwman et al., 2011). Phytate also poses nutritional challenges in livestock production. Since phytate

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P is not digestible by monogastric livestock, it cannot be absorbed and utilized for metabolic processes. Only P not bound to phytate is considered available P because it has a relatively high bioavailability (National Research Council, 2012). The available P present in cereals and legumes is not adequate for optimal animal growth and thus P must be supplemented in the diets of these animals or phytase enzymes must be added to the feed to break down phytic acid (Knowlton et al., 2004). Both add to feed costs, and supplemental P increases phosphorous run off.

Like other monogastric animals, humans do not produce significant quantities of phytase (Iqbal et al., 1994). There is some evidence that the human gut microflora can produce phytases, but the evidence is not strong (Markiewicz et al., 2013). While P deficiency is not of concern in humans, high phytate consumption contributes to deficiencies of microminerals, particularly Fe. Limited Fe absorption strongly contributes to Fe deficiency in developing countries where diets consist primarily of staple cereal crops rich in phytic acid (World Health Organization, 2001).

Human Fe absorption studies have examined the effects of dietary phytate, as pure Na phytate or as part of bran, on Fe bioavailability. Small amounts of Na phytate (2 mg phytate P) added to a phytate-free bread meal significantly inhibited Fe absorption. The inhibitory effect of increasing amounts of Na phytate resulted in a dose-dependent decrease in Fe absorption (Hallberg et al., 1989). In another study, addition of maize bran phytate to bread also showed a dose-dependent inhibition on Fe absorption (Siegenberg et al., 1991). In both of these studies, the meals were fortified with inorganic Fe. These results may not represent how phytate affects Fe naturally present in foods because of food matrix effects.

Because of the nutritional and environmental problems caused by phytate, *lpa* mutants of cereal crops and legumes have been developed. In maize, these mutants have a 30 to 90% reduction in phytate concentration and are generally accompanied by a molar equivalent increase in nonphytic acid P (Raboy, 2009). These mutants had greater Fe bioavailability, resulting in a 49% increase in Fe absorption (Mendoza et al., 1998) and improved P utilization in pigs (Hill et al., 2009). However, these mutants typically have poor seed germination, yield, or dry weight, making them undesirable for farmers (Raboy, 2009).

An alternative breeding approach to alter phytate levels in maize is through recurrent selection. This method involves selecting and intermating individuals with extreme values of a trait in a repeated cycle to create progeny with altered levels of the selected trait and has been successfully used to modify grain composition (Dudley and Lambert, 2010) and grain amino acid concentration (Scott et al., 2008). Phytate levels can change in populations under recurrent selection for oil concentration (Raboy et al., 1989), and phytic acid has the genetic

variation required to respond to selection (Lorenz et al., 2008). We therefore initiated a recurrent selection breeding program for phytic acid in maize.

Our research had three objectives. The first objective was to compare phytate levels in two populations selected for HPA or LPA concentration in maize. The second objective was to measure seed quality of *lpa* mutant maize and the HPA and LPA populations produced in objective one. The third objective was to measure the Fe bioavailability of the *lpa* mutants and the HPA and LPA maize populations produced in objective one.

MATERIALS AND METHODS

Breeding Strategy

Two broad-based synthetic maize populations were used in this study. BS31 is derived from FS8B and is largely tropical in origin (Horner, 1990), while BS11 is derived from the Pioneer Two-Ear Composite, a US Corn Belt population originally designed to be prolific (Hallauer, 1967). These populations were chosen because of their superior agronomic performance in our area and because they have responded to selection for other grain traits (Scott et al., 2008). Individually harvested ears from both the BS11 and BS31 populations were screened for relative phytate concentration via the Wade method (described in detail below). The five ears with the highest and five ears with the lowest relative phytate concentrations from each population were chosen to make balanced bulks of kernels, resulting in four populations each containing kernels from five ears: BS11HPA (BS11 high phytic acid), BS11LPA (BS11 low phytic acid), BS31HPA (BS31 high phytic acid), and BS31LPA (BS31 low phytic acid). The use of “high phytic acid” and “low phytic acid” here are population labels and do not refer to specific levels of phytic acid.

For the recurrent selection breeding, seeds from each of these four populations were planted in adjacent rows. The resulting plants were intermated in a chain-sib mating design to produce 50 ears from each of the four populations. These ears were harvested individually and screened for relative phytate concentration. The five ears with the highest relative phytate concentration from the BS11HPA category were made into a balanced bulk, as were the five ears with the lowest phytate concentration from the BS11LPA category, and the same was done for BS31HPA and BS31LPA. This process completed one cycle of recurrent selection. Three cycles were completed and the four cycle-three populations were used as seed for this study.

Evaluation of Recurrent Selection for Phytate Concentration

To determine the effectiveness of recurrent selection on phytate concentration, we produced grain from seed from four individually harvested ears from cycle three of each population as described below. An additional four ears consisting of inbred homozygous *lpa1-1* mutants, which typically have a 66% reduction in phytate concentration compared with wild-type maize (Raboy et al., 2000) were also included. Seed from each ear was planted in a one-row plot in a completely randomized block design with environment as a blocking factor. Thus we had five genotypes (four recurrent selection populations and the *lpa*

Table 1. Evaluation of relative standardized phytic acid (PA) concentration and standardized extractable P concentration for the high phytic acid (HPA) and low phytic acid (LPA) populations derived from BS11 and BS31, and *lpa* mutants. For PA, a smaller value implies a higher PA concentration. Means in the same column followed by different letters are statistically different ($P < 0.05$).

	Standardized PA [†]		Standardized extractable P [†]	
	BS 11	BS 31	BS 11	BS 31
HPA	−0.60a	−0.21a	−0.09a	−0.10a
LPA	0.04b	−0.05a	−0.08a	−0.07a
<i>lpa</i>	1.13c	1.13b	0.48b	0.48b

[†] For PA and extractable P, absorption at 490 and 820 nm, respectively, was converted to standardized data (mean of 0, standard deviation of 1). Units are standard deviation from mean.

mutants) replicated four times in each block. Six environments were used in the study. In 2010, the experiment was grown at the Bennett Farm, the Bruner Farm, and the Agronomy and Agricultural Research Farm near Ames, IA. In 2011, the experiment was grown at the Lippert Farm, the Burkey Farm, and the Agronomy and Agricultural Research Farm near Ames, IA. For each entry, three self-pollinations (subsamples) were made at each environment. At maturity, each ear was individually harvested and dried to approximately 150 g H₂O kg^{−1} fresh weight.

Measurement of Phytic Acid and Extractable Phosphorous for Recurrent Selection

Phytic acid and extractable P were measured using colorimetric assays modified from Vaintraub and Lapteva (1988) and Raboy et al. (2000) as described fully in Lorenz et al. (2007) using reagents obtained from Sigma-Aldrich. To facilitate high throughput screening and because ranking is sufficient for the purpose of selection, standards were not used. The phytate assay is unusual among colorimetric assays in that a lower phytic acid concentration is reflected by a higher absorbance because phytic acid decolors the Fe³⁺–sulfosalicylate complex in the Wade reagent (Vaintraub and Lapteva, 1988). The ears with the highest or lowest relative phytate concentration were chosen for HPA or LPA categories, respectively. Phytate and extractable P values were converted from absorbance to standardized values (mean 0, standard deviation 1) before data analysis to facilitate interpretation. This assay was used for evaluation of phytate levels in the recurrent selection program and to analyze the grain evaluated in this study. These results are presented in Table 1.

Seed Quality Evaluation: Standard Germination and Cold Tests

Seed for these tests was harvested by hand at physiological maturity or black layer formation (Hunter et al., 1991). Ears were immediately placed in a single layer inside a small-scale experimental dryer (Navratil and Burris, 1982) and air-dried with forced air at temperature below 35°C until the seed was at 120 g H₂O kg^{−1} fresh weight. Seed quality in this study is defined as the physiological seed characteristics that allow uniform and rapid seedling development (seed germination) and growth under a wide range of environmental conditions (seed vigor). To investigate seed

quality, we applied two measures that are detailed below: a standard germination test and a cold test. Ears were mechanically shelled individually and 100 seeds were counted from each ear for both the standard germination and cold tests.

Standard Germination Test

The standard germination test was used to evaluate seed viability under ideal growing conditions. The tests were conducted and percentage of normal seedlings were evaluated according to the Association of Official Seed Analysts (2010) rules for testing seeds. Each sample consisted of 100 seeds per ear and 43 to 69 ears per population. Samples were randomized within trays and germination carts, and four samples were planted in each tray. Seeds were planted on top of two sheets of crepe cellulose paper media (Kimberly Clark Corp.) moistened with 800 mL of tap water on fiberglass trays (45 by 66 by 2.54 cm). The trays were placed inside germination carts after planting, and the carts were placed inside a constant 25°C walk-in germination chamber with alternating 4 h of light and 4 h of darkness for a total of 12 h of light d^{−1}.

Cold Test

A 7-d cold test (Association of Official Seed Analysts, 2009) was used to evaluate seed vigor. Samples—45 to 69 per population—were randomized within trays and germination carts, and four samples were planted in each tray. One hundred seeds per ear were planted on one sheet of Kimpak (Kimberly Clark Corp.) watered with 1100 mL of water pre-chilled for 24 h at 10°C on fiberglass trays (45 by 66 by 2.54 cm). One centimeter of dry 80:20 sand/soil mixture was used to cover the seed and media. The trays were placed inside enclosed germination carts after planting, and the carts were placed inside a dark walk-in chamber at constant 10°C for 7 d and then moved to a constant 25°C walk-in germination chamber with alternating 4 h of light and 4 h of darkness for a total of 12 h of light d^{−1} for 7 d. Normal seedlings (Association of Official Seed Analysts, 2010) were evaluated and recorded.

Iron Bioavailability

Iron bioavailability was assessed for BS11HPA, BS11LPA, and *lpa* mutants (the LPA and HPA populations derived from BS31 were not assessed because there was no statistical difference in phytate concentration among the HPA and LPA populations). Balanced bulks from approximately 50 ears of each population were made from ground maize samples. To measure Fe bioavailability, samples were subjected to in vitro digestion followed by Fe uptake by Caco-2 cells as described below. Ferritin production in response to cell Fe uptake is used as an index for Fe bioavailability, which is expressed as nanogram of ferritin per milligram of cell protein (Glahn et al., 1998).

In Vitro Digestion

Samples and positive control were subjected to in vitro digestion as per Proulx and Reddy (2007) and modified as follows. The positive control consisted of 0.17 μmole FeSO₄ in 0.01 M hydrochloric acid (HCl) and was treated similar to the samples. All reagents used for in vitro digestion were purchased from Sigma-Aldrich. Ten milliliters of 0.01 M HCl was added to 1.75 g of maize flour along with 8.5 μmol ascorbic acid in 0.01 M HCl to increase Fe uptake response to detectable levels since

Table 2. Linear model of effects on standardized phytic acid concentration. Subsamples (1–3) were averaged before analysis.

	Phytic Acid		Extractable P		Std. Germ		Cold Germ	
	df	p-value	df	p-value	df	p-value	df	p-value
Environment (location and year)	5	0.0025**	5	<0.0001***	5	0.0009***	5	0.0042**
Genotype	4	<0.0001***	4	<0.0001***	4	<0.0001***	4	<0.0001***
G × E	20	0.9265	20	<0.0001***	20	0.0037**	20	0.0108*
Error	86		83		85		83	
Total	115	<0.0001***	112	<0.0001***	114	<0.0001***	112	<0.0001***
Outliers removed	3		5		3		4	
Model R^2	0.49		0.99		0.60		0.49	
Repeatability†	.26		.01		.47		.14	

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† Repeatability is defined as the proportion of the sums of squares due to genotype effects: $\text{Repeatability} = \sigma^2_{\text{genotype}} + [\sigma^2_{\text{G} \times \text{E}} / (\text{number of environments})] / \text{total } \sigma^2$

Fe concentration was low in the maize samples (Bodnar et al., 2013). The pH was adjusted to 2.0 with 1 M HCl, and then 520 μL of pepsin solution (0.2 g 1:60,000 porcine pepsin in 0.01 M HCl) was added and the samples were shaken in a water bath at 37°C for 1 h to simulate gastric digestion. The pH was then increased to 6.0 with 1 M sodium bicarbonate, and 1.3 mL of pancreatin and bile solution (0.05 g of 4×USP porcine pancreatin and 0.3 g of bile extract in 25 mL of 0.1 M sodium bicarbonate) was added and the samples were shaken again for 15 min at 37°C in a water bath to simulate duodenal digestion. The digests were centrifuged at 5000 g for 15 min, and the resultant supernatant was heated at 100°C for 4 min to deactivate digestive enzymes.

Caco-2 Cell Iron Uptake

The Fe uptake experiment was conducted with Caco-2 cells (American Type Culture Collection) as described fully in Bodnar et al. (2013). Reagents and other material were obtained from Sigma-Aldrich or Gibco BRL unless otherwise mentioned. Cells at passage 30 to 32 were used for experiments.

At 15 d postseeding, the cells were used for the Fe uptake experiment. Each digest was applied in duplicate in two separate wells on two separate cell culture plates (duplicates were averaged before analysis). Iron bioavailability was assessed as nanograms ferritin produced by cells in response to treatment per milligram cell protein.

Iron Analysis of Bulk Maize Samples

Nonheme Fe and non-protein-bound Fe were measured for bulks used in Fe bioavailability as well as single-kernel bulks made from self-pollinated ears from cycle three. For total non-heme Fe, trichloroacetic acid (TCA) treatment was followed by a colorimetric assay (Torrance and Bothwell, 1968). Maize flour (0.5 g) was mixed with 2 mL 10% TCA/3 N HCl and incubated for 20 h at 65°C to measure total Fe, including protein-associated Fe. The mixture was then centrifuged at 5000 g for 15 min, and the resultant supernatant was mixed with 0.02% ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt] in 2.05 M sodium acetate. The mixture was incubated for 10 min and the absorbance was measured at 563 nm. The protocol for non-protein-bound Fe

was identical, except that the maize flour was treated with TCA alone to precipitate proteins with bound Fe (Murray-Kolb et al., 2003). Protein-bound Fe was estimated by subtracting non-protein-bound Fe from total nonheme Fe that was measured with TCA and TCA plus HCl supernatants, respectively.

Data Analysis

To evaluate the self-pollinated ears that originated from the cycle three selections, one-way ANOVA followed by Tukey–Kramer multiple comparisons test were performed separately for both populations (BS11 and BS31) comparing the means of the HPA category, LPA category, and *lpa* mutants. This analysis was performed for phytic acid concentration, extractable P (Table 1), standard germination, and cold test (Table 3). Linear modeling was also performed for these same four outcome variables using JMP Pro 10 software (SAS Institute, 2013) to determine the effects of genotype (BS11HPA, BS11LPA, BS31HPA, BS31LPA, and the *lpa* mutants), environment (location and year), and the genotype × environment interaction (G × E) (Table 2). All effects in the linear models were modeled as fixed effects, limiting the scope of inference of this study to the treatments observed. Outliers were removed according to Anscombe and Tukey (1963). Subsamples of a given genotype–environment–replicate combination were averaged before analysis. For the Fe bioavailability results, one-way ANOVA followed by Tukey–Kramer multiple comparisons test was performed to compare the mean nanogram of ferritin per milligram of protein among three balanced bulks. Iron values of the same bulks used in the Fe bioavailability experiment as well as the single-kernel bulks were analyzed by ANOVA followed by Tukey–Kramer multiple comparisons.

RESULTS AND DISCUSSION

Relative Extractable Phosphorous and Phytic Aid Concentration in Recurrent Selection Populations

We performed two recurrent-selection experiments in two broad-based synthetic populations: one derived from BS11 and one derived from BS31. Divergent selection resulted in HPA and LPA populations. Because of reasonable precision, simplicity, and low cost to screen large number

of samples, the Wade method was recommended by the previous researchers (Lorenz et al., 2007). Concern was raised by Thavarajah and Thavarajah (2014) that the values obtained with the Wade method overestimated results compared with the more sensitive high-performance liquid chromatography (HPLC) method. However, good correlation (94–97%) was shown between Wade method and the widely used HPLC, anion exchange, and nuclear magnetic resonance methods (Gao et al., 2007, Lorenz et al., 2007). To alleviate concern about the method used for phytate measurement, we measured phytate levels in the samples used for Fe bioavailability analysis using a different, more quantitative method (see below) and these results are in good agreement with those presented here. After three cycles of recurrent selection, the LPA and HPA populations from the BS11 population had significantly different relative phytic acid concentration from each other and from the *lpa* mutants (Table 1; $P < 0.001$ for each pair). The *lpa* mutants were lowest, followed by LPA, with HPA having the highest relative phytic acid concentration. The *lpa* mutants also had significantly lower phytic acid levels than both the HPA and LPA populations in BS31 (Table 1; $P < 0.001$ for each pair). Comparison of phytic acid levels between the HPA and LPA populations of BS31 showed the LPA population was lower than the HPA population, but this difference was not significant (Table 1; $P > 0.05$). For this reason, we focused on the BS11-derived populations for the Fe bioavailability studies.

Both the BS31 and the BS11-derived LPA and HPA populations had significantly lower relative extractable P than the *lpa* mutants ($P < 0.05$; Table 1). However, the LPA and HPA populations were not significantly different from each other ($P > 0.05$; Table 1).

These results show that three cycles of recurrent selection were successful at significantly altering phytic acid in the BS11 population but not in the BS31 population. Although the results from this portion of the study were relative and not quantitative, it is evident that the phytic acid concentration of the BS11LPA was higher than the phytic acid concentration of the *lpa* mutant. Our recurrent selection approach allows one cycle of selection per year and it may take many more cycles of selection to determine if phytic acid concentration can be reduced to the levels seen in *lpa* mutants. These results also suggest that recurrent selection for reduced phytic acid concentration does not impact extractable P.

Although, in *lpa* mutants, P is repartitioned from phytate-bound P to nonphytate P; this may not be true for nonmutant maize, as evidenced by Lorenz et al. (2007, 2008). This observation is contrary to that of other maize families where phytate and nonphytate P are typically highly positively correlated (Raboy et al., 2001). Caution must be used when comparing our extractable P data to the total phosphate data reported in other studies, because

Table 3. Mean seed standard germination and cold test of the high phytic acid (HPA) and low phytic acid (LPA) populations derived from BS11, BS31, and *lpa* mutants. The standard germination test measures germination under optimal conditions and cold test evaluates seed vigor under cold conditions. Values are given as percentage of normal seedlings \pm SE.

	Standard germination test		Cold test	
	BS 11	BS 31	BS 11	BS 31
LPA	95a [†] \pm 1	90a \pm 2	89a \pm 3	92a \pm 2
HPA	95a \pm 2	90a \pm 2	86ab \pm 3	92a \pm 3
<i>lpa</i>	78b \pm 2	78b \pm 2	76b \pm 3	76b \pm 2

[†] Means within a column followed by different letters are statistically different ($P < 0.05$).

our extractable P method is designed for ranking samples and not for comparing with other values generated by other methods. The lack of extractable P differences between HPA and LPA populations suggests that phosphate may be repartitioned from phytate into other forms in populations selected for LPA.

The suitability of maize for animal feeds depends on digestible P concentration. While humans are generally at low risk for low P intake nutritional problems, the growth of livestock such as pigs can be limited by digestible P in their diet (Zhai and Adeola, 2013). Therefore, if digestible P in low-phytate maize is increased via recurrent selection, then the addition of supplemental P may be unnecessary.

We next determined the impact of genotype and environment on phytic acid concentration and extractable P. The linear models (Table 2) examined the impact of genotype, environment, and $G \times E$ interactions on standardized phytic acid and extractable P concentrations. The genotype and environment effects were significant. The environment effects are confounded with the laboratory analysis effects because the samples were not randomized across years in the laboratory analysis (the 2010 samples were analyzed in one batch and the 2011 samples were analyzed in another). The lack of a significant $G \times E$ effect for phytate suggests that it is not important to evaluate these genotypes in many environments to obtain an accurate ranking. This may in part explain our success in breeding for phytic acid levels, where selections were made based on data from a single environment. This is in contrast to extractable P, which had a significant $G \times E$ effect. Repeatability on the basis of an individual genotype was generally low because of the large environmental effects and the confounded laboratory measurement effects.

Seed Quality Evaluation

The germination percentage of LPA and HPA in the BS11-derived populations (95%) was significantly higher than the germination rate of the *lpa* mutants (78%; Table 3; $P < 0.0001$). Similar results were found in BS31, where LPA and HPA had the same germination (90%), which was significantly higher than that of the *lpa* mutants (Table 3; $P < 0.001$).

Table 4 Iron bioavailability (BA, ng ferritin mg⁻¹ cell protein), phytic acid (PA, mg PA g⁻¹ maize), and total nonheme (NH) Fe (μg Fe g⁻¹ maize), ± standard deviation, of bulked maize samples from the high phytic acid (HPA) and low phytic acid (LPA) populations derived from the BS11 population and *lpa* mutants. Iron bioavailability was performed in Caco-2 cell cultures and is expressed as ng ferritin mg⁻¹ cell protein. Four in vitro digestions were performed per sample, and each digest was applied to two cell wells (values from the two wells were averaged before analysis, *n* = 4). Ferrous sulfate was used as a positive control with an average value of 306.9 ng ferritin mg⁻¹ protein (*n* = 4). For Fe and phytate values, analyses were performed in duplicate except for BS11LPA, which was measured only once.

	Iron BA	PA	Total NH iron	NPB Fe	PB Fe
BS11LPA	26.7 ± 3.6a†	7.8a,b	16.1 ± 1.4b	11.2 ± 0.9a	4.9 ± 2.3a
BS11HPA	19.4 ± 3.0b	9.0 ± 0.1b	16.4 ± 0.0ab	12.4 ± 1.2a	4.0 ± 1.1a
<i>lpa</i>	31.0 ± 3.0a	6.4 ± 0.3a	19.7 ± 0.4a	11.0 ± 0.5a	8.7 ± 0.1a

† Means within a column followed by different letters are statistically different (*P* < 0.05).

In the BS11 population, the cold germination of LPA was 89%, better than the *lpa* mutant's cold germination of 76% (Table 3; *P* < 0.01). The cold germination of HPA (86%) had similar cold germination as both LPA and *lpa* (Table 3; *P* > 0.05). For the BS31 population, LPA and HPA had better cold germination (92%) than the *lpa* mutant (76%; Table 3; *P* < 0.0001).

To test the effects of environment and genotype on seed quality, the cold test and standard germination test data were fit to linear models (Table 2). For cold test, environment, genotype, and *G* × *E* were all significant (Table 2; *P* = 0.0042, *P* < 0.0001, and *P* = 0.0108, respectively) and the *R*² was 0.49. For the standard germination data, all three effects were also significant, with *P* = 0.0009 for environment, *P* < 0.0001 for genotype, and *P* = 0.0037 for *G* × *E* (Table 2). The *R*² for this model was 0.60. These results emphasize the importance of multi-environment trials for the evaluation of seed quality.

These results also indicate that recurrent selection did not have a negative impact on seed quality, and both the BS11-derived and BS31-derived LPA populations outperformed the *lpa* mutants. No other studies have examined the impact of phytate in nonmutant varieties on seed quality, but there is abundant evidence that *lpa* mutants of maize have poor seed quality. Early studies on *lpa 1-1* did not show a reduction in seed germination but did show a reduction in yield (Ertl et al., 1998). However, later experiments demonstrated a large reduction in seed germination and stress tolerance for *lpa1-1* and *2-1* mutants (Raboy, 2009). These mutants also have lower seed dry weight, an undesirable characteristic (Raboy et al., 2000; Shi et al., 2007). Other *lpa* mutants also have shown poor seed quality. The two mutants with the greatest reduction in phytate, approximately 90%, also have the greatest reduction in seed germination. The *lpa1-241* mutant had a 30% reduction in germination (Pilu et al., 2003) and the *lpa1-7* mutation was lethal when homozygous (Cerino Badone et al., 2012). Shi et al. (2005) reported that the *lpa3-1*, had good emergence under field conditions despite having a 50% reduction in phytic acid, but germination tests were not conducted. The success of our LPA populations developed with recurrent selection suggests that

further cycles of breeding may reduce phytate concentration while preserving seed quality.

Iron Bioavailability of Bulk Maize Samples

We next assessed if the observed differences in phytate levels corresponded to differences in Fe bioavailability. Iron bioavailability was significantly different among the three bulked maize samples (BS11LPA, BS11HPA, and *lpa*) tested. The mean for BS11LPA was 38% higher than for BS11HPA, and the *lpa* mutant was 60% higher than BS11HPA (*P* < 0.05; Table 4). There was no significant difference between BS11LPA and the *lpa* mutant (*P* > 0.05). These results indicate that even the relatively small difference we observed in phytic acid concentration can lead to a significant increase in Fe bioavailability in maize.

Phytate and Fe (total nonheme Fe, non-protein-bound, and protein-bound) were also quantitatively measured on the two BS11-derived populations, as well as the *lpa* mutants. Phytic acid analysis of the bulked maize samples showed a 29% decrease in phytic acid concentration of the BS11LPA bulk when compared with the BS11HPA bulk, while the *lpa* mutant bulk had 18% less phytic acid than the BS11LPA bulk (Table 4). The Fe analyses of these samples also indicate that there are differences in total Fe concentration among the three maize populations. The *lpa* mutant had approximately 20% greater total nonheme Fe than the other two populations (Table 4). In our study, 24 to 44% of Fe was estimated to be protein bound. Although there is no data on the Fe distribution in staple crops, one study aimed at looking at Fe absorption in humans reported that soybeans [*Glycine max* (L.) Merr.] have 49% of the Fe as protein bound (Murray-Kolb et al., 2003). Our data suggest that *lpa* mutants have higher bioavailability not only due their LPA concentration but also due to their Fe bound to protein, presumably to ferritin, which is more bioavailable (Lonnerdal et al., 2006).

To further investigate the Fe speciation in the seeds, a series of five single-kernel bulks for each of the three maize populations was created. While no difference in total Fe was found between the three populations (*P* > 0.05; Fig. 1), a significant difference was found in non-protein-bound Fe between *lpa* and each of the BS11

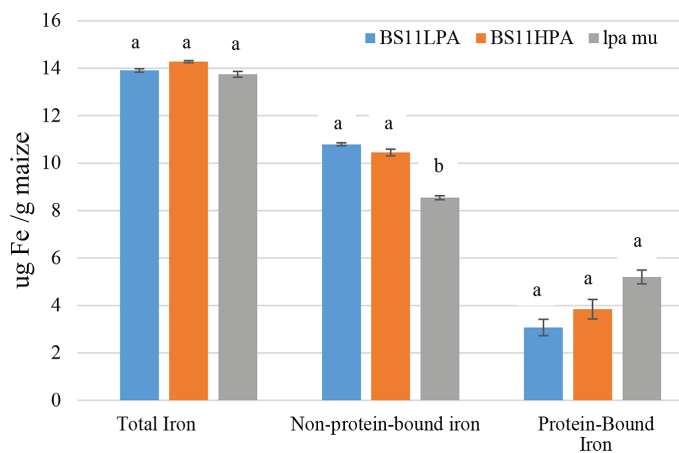


Figure 1. Iron concentration and speciation of bulks made from high phytic acid (HPA) and low phytic acid (LPA) populations of maize derived from the BS11 population and *lpa* mutants. Bars are mean \pm SE. Bars with different letters are significantly different ($P < 0.05$, $n = 5$).

populations (Fig. 1; $P < 0.01$ for BS11LPA and $P < 0.05$ for BS11HPA), but there was no difference between BS11LPA and BS11HPA ($P = 0.929$).

The difference in Fe bioavailability between the *lpa* mutants and the BS11-derived populations is likely attributable to a reduction in phytic acid concentration accompanied by an increase in protein-bound Fe. Because of the toxicity of free Fe in plants, Fe is typically found bound to phytate or the protein ferritin (Morrissey and Guerinot, 2009). Therefore, in response to a reduction in phytate the maize plant would produce seed with more ferritin to limit the amount of free Fe. The bioavailability of ferritin in humans is reported to be approximately 50 (Murray-Kolb et al., 2003) to 100% (Lonnerdal et al., 2006) of ferrous sulfate. A study in which *lpa1-1* maize mutants were transformed to contain the soybean ferritin gene found that Fe bioavailability in Caco-2 cells could be doubled by the addition of the ferritin transgene. This study also found that nontransgenic *lpa1-1* mutants have a 50% increase in ferritin when compared with nonmutant seeds (Aluru et al., 2011). These findings suggest that ferritin in maize should be a bioavailable source of Fe. If the increase in protein-bound Fe found in the *lpa1-1* mutants is in fact ferritin bound, we would have seen a much higher Fe bioavailability in the *lpa1-1* mutants in the present study. More research should be conducted to determine if *lpa1-1* mutants are in fact higher in ferritin than the BS11LPA maize or other factors that would explain why *lpa1-1* did not have higher Fe bioavailability.

These results are promising for the future of low-phytate crops. In contrast to previous studies that suggest that phytate must be almost completely removed before an increase in Fe bioavailability occurs (Hurrell et al., 1992), this study suggests that it may be possible to increase Fe bioavailability with only relatively minor reductions in

phytate. This study demonstrates that recurrent selection can be used to produce low-phytate maize with improved Fe absorption. More studies should be done to determine the optimal amount of phytic acid reduction in maize required to maximize Fe bioavailability.

CONCLUSIONS

This study concludes that recurrent selection can be used to alter grain phytic acid concentration in maize without negatively affecting seed quality, making recurrent selection an attractive alternative to altering phytic acid levels with *lpa* mutants. Moreover, selection for phytic acid levels may result in altered Fe bioavailability, demonstrating that even small decreases in phytic acid concentration can significantly improve Fe bioavailability. We conclude that recurrent selection for phytic acid levels may be an effective approach to improving Fe bioavailability of grain while preserving seed quality.

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